

# Activation of Bone Marrow Phagocytes Following Benzene Treatment of Mice

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Techniques in flow cytometry/cell sorting were used to characterize the effects of benzene and its metabolites on subpopulations of bone marrow cells. Treatment of male Balb/c mice with benzene (880 mg/kg) or a combination of its metabolites, hydroquinone and phenol (50 mg/kg), resulted in a 30 to 40% decrease in bone marrow cellularity. Flow cytometric analysis revealed two subpopulations of bone marrow cells that could be distinguished by their size and density or granularity. The larger, more dense subpopulation was found to consist predominantly of macrophages and granulocytes as determined by monoclonal antibody binding and by cell sorting. Benzene treatment had no selective cytotoxic effects on subpopulations of bone marrow cells. To determine if benzene treatment activated bone marrow phagocytes, we quantified production of hydrogen peroxide by these cells using the fluorescent indicator dye, 2',7'-dichlorofluorescein diacetate. We found that macrophages and granulocytes from bone marrow of treated mice produced 50% more hydrogen peroxide in response to the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate than did cells from control animals. It is hypothesized that phagocyte activation and production of cytotoxic reactive oxygen intermediates may contribute to hematotoxicity induced by benzene.

## Introduction

Benzene is a potent bone marrow toxin. Acute treatment of experimental animals with benzene or its metabolites results in a decrease in bone marrow cellularity, as well as in impaired hematopoiesis and immune system functioning (1-7). The mechanisms underlying these effects are unknown, but may involve a direct action of benzene or its metabolites on specific cellular components of the bone marrow.

Recently, it has been proposed that bone marrow stromal cells, in particular, macrophages, may represent a primary cellular target for benzene (8-11). These cells are known to release a variety of cytokines and growth factors that modulate the proliferation and activation of stem cells (12,13). Following tissue injury or antigenic stimulation, bone marrow macrophages, like other mononuclear phagocytes, become activated. These cells display enhanced functional and biochemical responsiveness and release elevated levels of cytokines (14). Activated phagocytes also produce highly reactive and potentially toxic oxygen intermediates including superoxide anion, hydrogen peroxide, and hydroxyl radical (14-17). Activation of stromal phagocytes in the bone marrow following benzene exposure with associated alterations in

the release of growth regulatory molecules and/or reactive oxygen intermediates may have profound effects on stem cell proliferation and differentiation.

The present studies were designed to determine if benzene treatment of mice activates bone marrow phagocytes to produce elevated levels of reactive oxygen intermediates. For these studies, we used techniques in flow cytometry/cell sorting. The advantage of this technology is that it permits rapid and precise analysis of cellular activation on a single cell basis from a mixed population of bone marrow cells. We found that benzene treatment of mice depleted the number of recoverable bone marrow cells. Accompanying this response, the toxin was found to cause specific activation of bone marrow macrophages and granulocytes. Thus, these cells produced elevated levels of hydrogen peroxide. These results support the hypothesis that benzene induced phagocyte activation and production of cytotoxic reactive oxygen intermediates may contribute to hematotoxicity.

## Materials and Methods

### Treatment of Mice

Male Balb/c mice (Taconic Farms), 20 to 30 g, were used in all experiments. Animals were injected SC with 880 mg/kg benzene or a combination of 50 mg/kg phenol and hydroquinone once per day for 3 days. Control animals received corn oil or phosphate-buffered saline (PBS).

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## Isolation of Bone Marrow Phagocytes

Control and treated animals were killed by cervical dislocation 24 hr after the last injection. The lower leg bones were then removed from the animals, and the femur and tibia were flushed with 5 mL of Hank's balanced salt solution (HBSS) to isolate bone marrow cells. Cell suspensions were then washed twice with HBSS and contaminating red blood cells lysed using 0.75% ammonium chloride in 20 mM Tris-HCl buffer (pH 7.2). Cells were enumerated using a Coulter counter.

## Indirect Immunofluorescence

To identify subpopulations of bone marrow cells, we used the highly specific monoclonal antibody, Mac-1 (Hybritech), which binds to mouse macrophages, monocytes, and granulocytes. Bone marrow cells ( $1 \times 10^6$ ) were incubated with a 1:100 dilution of Mac-1 antibody at 4°C. After 30 min, the cells were washed three times with PBS containing 0.1% gelatin and incubated for an additional 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated goat-F(ab')<sub>2</sub> anti-rat IgG (Jackson Labs). The percentage of positive cells binding the Mac-1 antibody was then determined by quantifying cell-associated fluorescence at 515 to 550 nm using a Coulter Epics 753 dye laser flow cytometer/cell sorter equipped with two 5-watt argon lasers. For each analysis, 20,000 events were recorded.

## Measurement of Phagocyte Oxidative Metabolism

Hydrogen peroxide production by bone marrow phagocytes was monitored using the indicator dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Inc.) as previously described (15-18). DCFH-DA is rapidly taken up by cells and cleaved by intracellular esterases to yield the nonfluorescent compound, DCFH. Hydrogen peroxide produced by activated phagocytes hydrolyzes DCFH to the fluorescent compound, 2',7'-dichlorofluorescein (DCF). Thus, the intensity of cell-associated DCF fluorescence is directly proportional to the amount of hydrogen peroxide produced by the cells. Bone marrow cells ( $1 \times 10^6$ ) were incubated for 15 min at 37°C with DCFH-DA followed by incubation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or control for 20 min. Green fluorescence associated with hydrogen peroxide production was then detected at 515 to 550 nm on the flow cytometer. Each experiment was repeated at least three times with similar results.

## Results

### Effects of Benzene Treatment on Bone Marrow Cellularity

Treatment of mice with benzene or a combination of hydroquinone and phenol resulted in a 30 to 40% decrease in the number of cells recovered from the bone marrow.

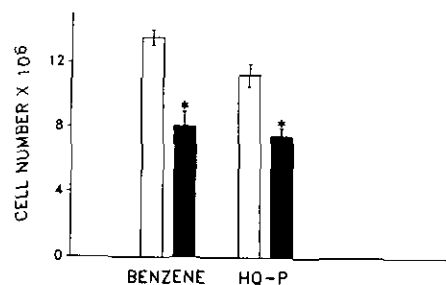


FIGURE 1. Effects of benzene and hydroquinone-phenol treatment of mice on bone marrow cellularity. Mice were injected with benzene (880 mg/kg), a combination of hydroquinone and phenol (50 mg/kg) or control (corn oil or PBS) once per day for 3 days. Cells were then isolated from the lower leg bones, washed, and counted. Each bar represents the mean  $\pm$  SE from 3 to 4 animals. Statistically significant ( $p \leq 0.05$ ) differences between control and treated animals are shown by asterisks (\*) (Student's *t*-test).

(Fig. 1). Differential staining of these samples revealed no obvious signs of toxicity as determined by light microscopy and by trypan blue dye exclusion (not shown). In addition, there appeared to be no selective toxicity of these compounds toward bone marrow cell subpopulations. Thus, the percentage of mature and precursor cells recovered from the bone marrow following benzene treatment remained the same.

### Characterization of Bone Marrow Cells by Flow Cytometry and Antibody Binding

Initially, we characterized subpopulations of cells isolated from the bone marrow of control mice and compared them to cells from animals treated with benzene or a combination of hydroquinone and phenol. Using flow cytometry, we could distinguish between two distinct subpopulations of bone marrow cells that differed with respect to size and density as determined by the forward and right angle light scatter properties of the cells (Fig. 2). These consisted of a larger, more dense subpopulation

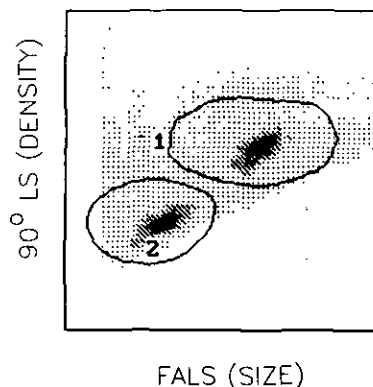


FIGURE 2. Identification of subpopulations of bone marrow cells by flow cytometry. The figure shows the results of analysis of bone marrow cells based on their laser light scatter properties. Cells scatter light in the forward angle direction (forward angle light scatter, FALS) according to their size and in the right angle direction (90° light scatter, 90° LS) according to their density or granularity.

(population 1) and a smaller, less dense population (population 2). Treatment of mice with benzene or its metabolites had no effect on the light scatter properties of the bone marrow cells (not shown).

To further characterize these subpopulations, we used the monoclonal antibody, Mac-1. This antibody binds to the CR3 receptor on mature mouse macrophages and granulocytes. By indirect immunofluorescence and flow cytometry, we found that only subpopulation 1 bound the Mac-1 antibody (Fig. 3). Sorting and microscopic examination of this subpopulation confirmed that it consisted predominantly of macrophages, monocytes, and granulocytes (not shown). Benzene had no effect on Mac-1 binding to the cells, confirming that the proportion of macrophages and granulocytes in the bone marrow was unaffected by treatment of the mice (not shown).

### Production of Hydrogen Peroxide by Bone Marrow Phagocytes

To determine if macrophages and granulocytes from bone marrow of benzene-treated mice produced elevated

levels of reactive oxygen intermediates, we monitored hydrogen peroxide production by these cells using DCFH-DA. In these experiments, a map or gate was drawn around population 1, the Mac-1 positive cells (Figs. 2 and 3.) Hydrogen peroxide production by this subpopulation was then quantified. We found that basal levels of hydrogen peroxide production by Mac-1 positive cells from control and treated mice were similar (Fig. 4). In contrast, phagocytes (Mac-1 positive cells) from benzene treated mice produced 50% more hydrogen peroxide in response to the phorbol ester tumor promoter, TPA, than did cells from control animals (Fig. 4).

Similar results were observed with cells obtained from the hydroquinone-phenol treated mice (not shown). In separate studies, we also found that the production of hydrogen peroxide by bone marrow phagocytes in response to TPA was inhibited by pretreatment of the cells for 5 min with 1  $\mu$ M catalase (not shown). These results demonstrate that bone marrow phagocytes from benzene-treated mice are activated.

### Discussion

Activated phagocytes are characterized by altered morphology, enhanced functional capacity, and release of reactive mediators. Although it is generally assumed that this results in more rapid destruction of foreign antigens, recent evidence suggests that the release of reactive mediators from phagocytes may also contribute to tissue injury (19,20). In the present studies we characterized macrophages and granulocytes isolated from bone marrow of mice treated with benzene, or its metabolites, hydroquinone and phenol, and determined if they were activated. We found that acute benzene treatment reduced the total number of cells in the bone marrow. However, the percentage of different subpopulations of cell types in the bone marrow remained the same as de-

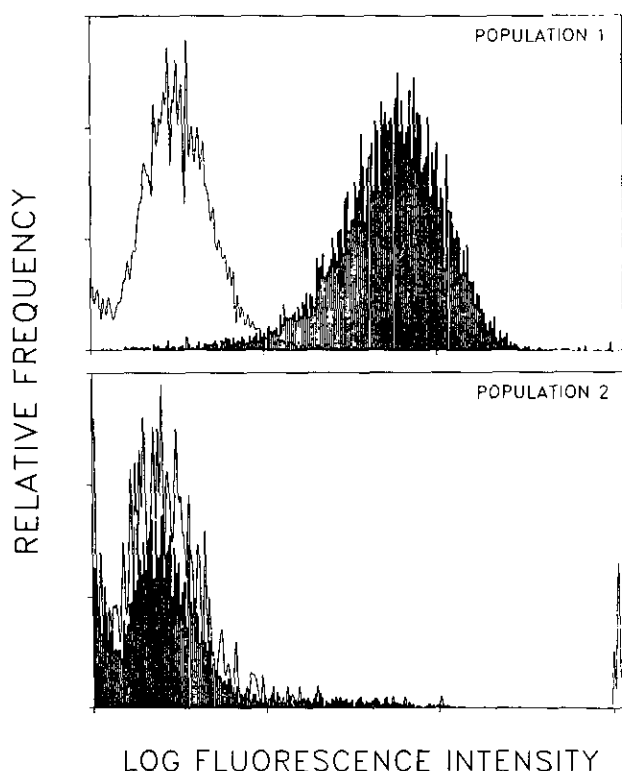


FIGURE 3. Binding of the anti-phagocyte monoclonal antibody, Mac-1 to subpopulations of bone marrow cells. Bone marrow cells isolated from control and treated mice were incubated for 30 min at 4°C with Mac-1 antibody followed by incubation with FITC-labeled goat antirat IgG. Binding of Mac-1 antibody to subpopulations of bone marrow cells, identified by differences in size and density as shown in Fig. 2, was then quantified by flow cytometry. The data are presented as log green fluorescence intensity, which is directly proportional to the amount of Mac-1 antibody binding, versus frequency or cell number. An increase in fluorescence intensity represents antibody binding to the cells.

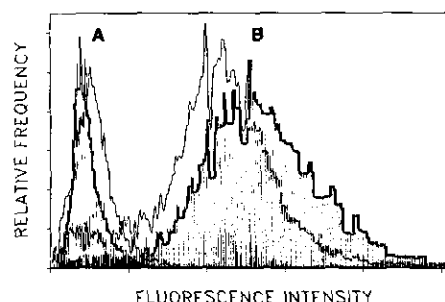


FIGURE 4. Stimulation of hydrogen peroxide production by bone marrow phagocytes following benzene treatment of mice. Bone marrow cells from control (unshaded curves) or benzene-treated (shaded curves) mice were incubated with the hydrogen peroxide indicator dye, DCFH-DA for 15 min and then stimulated with A, control (HBSS) or B, TPA (170 nM) for 20 min. The cells were then separated by flow cytometry on the basis of size and density as shown in Fig. 2. A gate was set around the Mac-1 positive cells (population 1) and hydrogen peroxide production by this subpopulation quantified on the basis of fluorescence intensity. The data are presented as fluorescence intensity, which is directly proportional to hydrogen peroxide production in these cells, versus frequency or cell number.

terminated morphologically and by Mac-1 binding. This suggested that the cytotoxic effects of benzene on bone marrow cells were not selective. In addition, although there were no obvious morphological differences between the bone marrow cell types as determined by light microscopy, phagocytes from benzene-treated mice produced 50% more hydrogen peroxide following stimulation with TPA than did cells from control mice and thus, may be considered activated.

Reactive oxygen intermediates such as superoxide anion and hydrogen peroxide have been implicated as primary mediators of macrophage-induced cellular injury and may play a role in benzene-induced cytotoxicity. Following stimulation, activated phagocytes produce superoxide anion, which can react with water and other molecules to generate hydroperoxy- and hydroxyl radicals (19,20). These radicals are highly toxic and can initiate membrane lipid peroxidation reactions and induce direct damage to cellular macromolecules. Changes in membrane lipid composition such as those induced by lipid peroxidation can lead to alterations in cell membrane permeability, disruption of cellular homeostasis, and eventually to cell death. Thus, the decrease in bone marrow cellularity observed following benzene exposure may be mediated, at least in part, by oxygen radicals generated from activated bone marrow phagocytes.

Bone marrow macrophages activated by benzene have also been reported to release elevated levels of interleukin-1, as well as other cytokines that can stimulate proliferation and differentiation of bone marrow stem cells and stromal cell production of growth factors (11). This can lead to increased proliferation of subpopulations of stem cells that may contribute to the development of benzene-induced leukemia.

Based on these results, we propose a model of benzene-induced bone marrow toxicity (Fig. 5). According to our model, phagocytes (macrophages and granulocytes) in the bone marrow become activated following exposure of the animal to benzene and/or its metabolites. Activation may occur by direct interaction of these compounds with the phagocytes or indirectly by products released from damaged bone marrow tissue and cells. These activated phagocytes produce inflammatory mediators such as superoxide anion and hydrogen peroxide that may be cytotoxic.

Immune mediators (IM) and cytokines are also released by activated phagocytes and these may stimulate or inhibit growth of subpopulations of bone marrow stem cells. Decreased cell proliferation may lead to acute bone marrow depression while enhanced cell proliferation may lead to leukemia. Thus, according to our model, activated bone marrow phagocytes may contribute to both the acute and long-term hematotoxic actions of benzene and its metabolites. The data presented in this paper together with those reported previously by other laboratories (9,11) support this hypothesis. Based on our model we would predict that activated phagocytes in the bone marrow can interact with and directly damage stem cells. Cytotoxicity studies with benzene-activated bone marrow macrophages are in progress to explore this possibility.

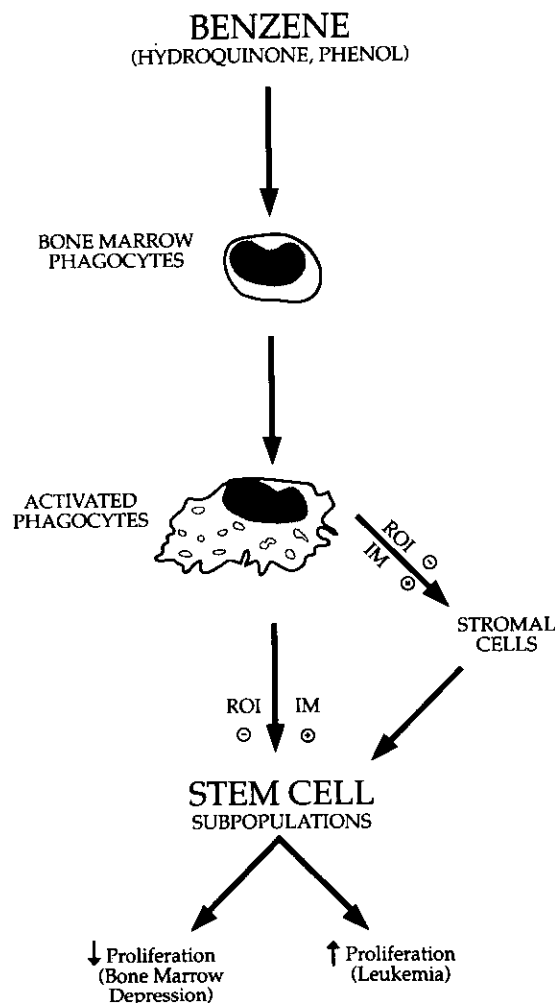


FIGURE 5. Model for the potential role of bone marrow phagocytes in benzene-induced hematotoxicity. Toxic doses of benzene induce activation of bone marrow phagocytes (macrophages and granulocytes). These activated phagocytes release reactive oxygen intermediates (ROI) that are cytotoxic toward stem cells and other stromal cells resulting in bone marrow depression. Activated phagocytes can also produce elevated levels of immune mediators (IM) and cytokines including IL-1 that alter the proliferation of subpopulations of bone marrow cells and this may be important in leukemogenesis.

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## REFERENCES

1. Eastmond, D. A., Smith, M. T., and Irons, R. D. An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. *Toxicol. Appl. Pharmacol.* 91: 85-95 (1987).
2. Irons, R. D., Neptun, D. A., and Pfeiffer, R. W. Inhibition of lymphocyte transformation and microtubule assembly by quinone metabolites of benzene. *J. Reticuloendothel. Soc.* 30: 359-371 (1981).
3. Pfeiffer, R. W., and Irons, R. D. Inhibition of lectin-stimulated lymphocyte agglutination and mitogenesis by hydroquinone: Reactivity with intracellular sulfhydryl groups. *Exp. Mol. Pathol.* 35: 189-198 (1981).
4. Snyder, R., and Engelsberg, B. N. Effects of benzene and other chemicals in 72 hr  $^{59}\text{Fe}$  utilization in Swiss albino mice. *Toxicologist* 2: 121 (1982).

5. Tunek, A., Olofsson, T., and Berlin, M. Toxic effects of benzene and benzene metabolites on granulopoietic stem cells and bone marrow cellularity in mice. *Toxicol. Appl. Pharmacol.* 59: 149-156 (1981).
6. Weir, D., and Irons, R. D. Hydroquinone and catechol reduce the frequency of progenitor B lymphocytes in mouse spleen and bone marrow. *Immunopharmacology* 4: 41-54 (1982).
7. Rosenthal, G. J., and Snyder, C. A. Inhaled benzene reduces aspects of cell mediated tumor surveillance in mice. *Toxicol. Appl. Pharmacol.* 88: 35-43 (1988).
8. Gaido, K. W., and Wierda, D. Suppression of bone marrow stromal cell function by benzene and hydroquinone is ameliorated by indomethacin. *Toxicol. Appl. Pharmacol.* 89: 378-390 (1987).
9. Schlosser, M. J., and Kalf, G. F. Activation of phenol and hydroquinone to covalently binding metabolites by mouse macrophage lysates. *Toxicologist* 8: 73 (1988).
10. King, A., Landreth, K., and Wierda, D. Hydroquinone inhibits macrophage regulation of stromal cell dependent B-lymphopoiesis. *Toxicologist* 8: 72 (1988).
11. Thomas, D. J., and Wierda, D. Bone marrow stromal macrophage production of interleukin-1 activity is altered by benzene metabolites. *Toxicologist* 8: 72 (1988).
12. Dorshkind, K. Bone marrow stromal cells and their factors regulate B-cell differentiation. *Immunol. Today* 8: 191-193 (1987).
13. Trenton, J. J. Influence of haematopoietic organ stroma (haematopoietic microenvironment) on stem cell differentiation. In: *Regulation of Haematopoiesis*, Vol. 1 (A. S. Gordon, Ed.), Meredith, New York, 1970, pp. 161-173.
14. Adams, D. O., and Hamilton, T. A. The cell biology of macrophage activation. *Annu. Rev. Immunol.* 2: 283-318 (1984).
15. Pilaro, A. M., and Laskin, D. L. Accumulation of activated mononuclear phagocytes in the liver following lipopolysaccharide treatment of rats. *J. Leukocyte Biol.* 40: 29-41 (1986).
16. Laskin, D. L., and Pilaro, A. M. Potential role of activated macrophages in acetaminophen hepatotoxicity. I. Isolation and characterization of activated macrophages from rat liver. *Toxicol. Appl. Pharmacol.* 86: 204-215 (1986).
17. Laskin, D. L., Robertson, F. M., Pilaro, A. M., and Laskin, J. D. Activation of liver macrophages following phenobarbital treatment of rats. *Hepatology* 8: 1051-1055 (1988).
18. Bass, D. A., Olbrantz, P., Szejda, P., Seeds, M. C., and McCall, C. E. Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. *J. Immunol.* 136: 860-866 (1986).
19. Cerutti, P. A. Prooxidant states and tumor promotion. *Science* 227: 375-381 (1985).
20. Babior, B. M. Oxidants from phagocytes. Agents of defense and destruction. *Blood* 64: 959-966 (1984).
21. Laskin, D. L., Pilaro, A. M., and Ji, S. Potential role of activated macrophages in acetaminophen hepatotoxicity. II. Mechanism of macrophage accumulation and activation. *Toxicol. Appl. Pharmacol.* 86: 215-226 (1986).